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Destruction of *Salmonella* Enteriditis inoculated onto raw almonds by high hydrostatic pressure

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Abstract

The effects of continuous (50,000, 60,000 and 70,000 psi with holding times of 5 and 10 min) and discontinuous (oscillatory) (six cycles at 60,000 psi with a holding time of 20 s) high hydrostatic pressure (HHP) treatments on the viability of two *Salmonella* Enteriditis strains (FDA and PT30) inoculated onto raw almonds were evaluated at 25, 50, and 55 °C. Complete inactivation of the *S.* Enteriditis was achieved in 0.1% peptone water after continuous pressurization at 60,000 psi and 25 °C for 5 min. Continuous pressurization of raw almonds inoculated with *S.* Enteriditis at 60,000 psi and 50 °C for 5 min resulted in less than a log reduction ($\log_{10}$ 0.83) of vegetative cells. The decimal reduction time using the continuous pressurization parameters was determined to be 9.78 min. A discontinuous process consisting of six cycles of pressurization at 60,000 psi and 50 °C for 20 s provided greater than a one log reduction ($\log_{10}$ 1.27 for FDA and $\log_{10}$ 1.16 for PT30) of the *S.* Enteriditis concentration. The low water activity (a_w) of the almonds was found to impart baroprotective attributes on the *S.* Enteriditis cells. When the almonds were directly suspended in water and then pressurized, a $\log_{10}$ reduction of 3.37 was achieved. HHP of certain dry foods appears to be feasible if the food is directly suspended in the pressurizing medium (water).

Keywords: Raw almonds; *Salmonella* Enterditis; Contamination; Outbreaks; High hydrostatic pressure; Decontamination; Low water activity; Baroprotective; Water pressurization

1. Introduction

High hydrostatic pressure (HHP) processing is a technique that is currently receiving a great deal of interest as a method to destroy pathogenic and spoilage microorganisms in foods. In the HHP process, the food (liquid or solid) is subjected to pressures as high as 130,500 psi (San Martin, Barbosa-Cánovas, & Swanson, 2002). The actual pressurization is carried out in a pressure vessel, containing a fluid that acts as the pressure transmitting medium. During the pressurization process, the pressure is applied isostatically, which allows the food to retain its original shape. The pressure is held for the desired time at the desired temperature and then released. The pressure, time, and temperature combination will depend on a number of factors including the type of food, and types of microorganisms to be destroyed.

HHP (14,500–101,500 psi) of foods at low or moderate temperature is being increasingly investigated as a non-thermal processing method to reduce the microbial load in foods. Bacterial cells, yeasts and molds are relatively sensitive to pressurization below 100,000 psi, but bacterial spores can be quite resistant. Several variables, including magnitude of pressure, pressurization time and temperature, microbial types, cell growth phase, suspending media, and the presence of antimicrobial substances have been reported to influence the hydrostatic pressure induced death of microbial cells (Alpas et al., 1999; Benito, Ventoura, Casadei, Robinson, & Mackey, 1999; Kalchayanand, Ray, Sikes, & Dunne, 1998a; Kalchayanand, Sikes, Dunne,

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& Ray, 1998b; Patterson & Kilpatrick, 1998; Palou et al., 1998). Generally, microbial destruction will increase with increases in pressure, pressurization time and temperature, in suspending media with low solid content, and in the presence of antimicrobial properties (Alpas et al., 1999).

Since 2001, raw whole almonds have emerged as a source of *S.* Enteriditis infection. In 2001, raw almonds were linked to an outbreak of *S.* Enteriditis infections, mostly in Canada, during a 6-month period (Isaacs et al., 2005). The *S.* Enteriditis had a rare phage type (PT30) which aided in identification of the outbreak strain. Recently, a second outbreak of a rare phage type of *S.* Enteriditis (PT9C) due to contaminated raw almonds was identified (CDC, 2004). This outbreak occurred in multiple US states and Canadian provinces, and was traced to a large commercial producer of almonds. Ultimately a large and ongoing international recall of the raw almonds ensued.

The recent outbreaks of salmonellosis infection due to consumption of contaminated raw almonds have necessitated development of effective processing methods to control the growth of this pathogen on raw almonds, without destroying the texture and sensory attributes of the raw almonds. The objective of this study was to determine whether HHP could be used as a processing method to reduce *S.* Enteriditis contamination of raw almonds.

2. Materials and methods

2.1. Bacterial strains

Two *Salmonella* Enteriditis strains were used in this study. *S.* Enteriditis strain FDA was chosen from our strain collection because it was previously shown to be very resistant to HHP (Alpas et al., 1999). *S.* Enteriditis PT30 was the isolate implicated in the almond outbreak in 2000–2001, and was obtained from the National Food Laboratory in Dublin, California. Stock bacterial cultures were maintained in 30% glycerol and were frozen at −70 °C.

2.2. Isolation and preparation of Salmonella strains

Prior to each experiment, bacterial strains were prepared according to Danyluk, Uesugi, and Harris (2005). For growth experiments, the inocula consisted of stationary phase cells that were obtained by inoculating tryptic soy broth (TSB) with a single colony from an overnight TSA plate and incubating the preparations overnight with shaking at 37 °C. The overnight (18 h) culture was used to inoculate 150 mm × 15 mm TSA plates to produce a bacterial lawn, following incubation for 24 h at 35 °C. Three plates were prepared per 400 g almond sample. Following incubation, approximately 8–9 ml of 0.1% peptone was added to each large plate. The bacterial lawn was loosened with a sterile spreader and cells (approximately 25 ml) were collected. Inoculum levels were determined by serial dilution in 0.1% peptone followed by plating onto TSA and XLD agar.

2.3. Inoculation procedures

Almond samples (400 ± 1 g) were weighed into plastic polyethylene bags (30.5 cm × 30.5 cm) (Bitran, Com-Pac Int., Carbondale, IL), followed by addition of 25 ml (10^9 CFU/ml) of the pooled inoculum. This volume had been previously determined to be the least needed to completely coat the almonds with little residual liquid remaining. The bag was closed and shaken by hand through inversion for 60 s. Almonds were poured out of the bag and spread onto two sheets of 46 × 57-cm filter paper (Fisherbrand Qualitative P8, Fisher Scientific, Pittsburgh, PA) to dry for approximately 1 h.

2.4. Enumeration procedures

Fifty grams of almonds were added to an equal volume of Butterfield's phosphate buffer. The almonds were stomached for 2 min in a LabBlender 400 (Seward Laboratory, London, UK) on normal speed. Serial dilutions were made in Butterfield's phosphate buffer followed by spiral plating onto TSA and appropriate selective (XLD) agar, with the use of an Autoplate 4000 spiral plate system (Spiral Biotech, Norwood, MA). After overnight incubation at 37 °C, the plates were enumerated using an automated Q colony counter (Spiral Biotech), and the software associated with the instrument.

2.5. Determination of water activity

The water activity of the almonds was determined using an AquaLab water activity meter (Decagon Pullman, WA). Before inoculation with the respective *Salmonella* strain, almonds were mixed and randomly sampled by placing groups of 4–5 almonds into three individual containers. The containers were covered and the almonds were allowed to equilibrate for 25–30 min. The AquaLab meter was calibrated using a commercially available standard, and the reproducibility of the AquaLab readings was assessed by taking two readings of an identical volume of deionized water. The water activity of the almonds was determined according to manufacturer's instructions. Following bacterial inoculation, the water activity of the almonds was determined after 1, 2 or 3 h of drying, storage overnight (8 h), and 24 h.

2.6. High hydrostatic pressure processing

High pressure experiments were carried out in a unit that consisted of an internal pressure chamber with dimensions of 10 cm × 24.5 cm length (Engineered Pressure Systems, Andover, MA, USA), and had the capability of pressurizing to 100,000 psi at 25–95 °C. The pressure chamber was filled with a liquid mixture of water and 5% oil

(Mobil Hydrosol 78) pumped into the bottom. Prior to pressurization, the liquid was heated to the desired temperature by electric heating coils surrounding the chamber. After chamber closing, additional liquid was pumped into the instrument through a check valve to generate the desired hydrostatic pressure level. A microprocessor controlled and displayed readings of pressure level, time and temperature of pressurization. Almond samples were pressurized at different pressures, temperatures, and times, depending on the study, as described below. These pressure/time/temperature combinations had been previously described in the literature, and have been used in hydrostatic pressure studies in various foods (Alpas et al., 1999; Kalchayanand et al., 1998a). Pressure-come-up time was about 3 min for every 50,000 psi, and come down time was within 1 min, depending on the pressure. The pressure increased in an isostatic manner (same value everywhere in the vessel and throughout the food sample). The pressure come-up and come-down times, as well as adiabatic temperature during pressurization (approximately 3 °C per 14,500 psi) were not used in the calculations of the pressurization time and temperature.

2.7. Pressurization of S. Enteriditis in pure culture

Lag phase TSB cultures of *S.* Enteriditis FDA and PT30 were diluted 10-fold in 0.1% peptone solution (pH 7.0), and transferred in 3 ml portions into sterile cryovials (3 ml capacity; Simport, Quebec, Canada). Each vial was vacuum sealed in plastic bags (Nasco, Ft. Atkinson, WI) and pretempered to either 25 °C or 50 °C for 5 min. The vials, in duplicate for each study parameter, were placed inside the pressure chamber of the pressure unit. HHP of the cultures was performed in a continuous manner for 5 min using pressure/temperature combinations of 50,000 psi/25 °C, 60,000 psi/25 °C, 50 °C, and 70,000 psi/25 °C.

2.8. Pressurization of S. Enteriditis inoculated almonds

2.8.1. Continuous process

Inoculated almonds, prepared as described, were weighed into 50 g portions, vacuum sealed in plastic bags, and pretempered to 25 °C, 50 °C or 55 °C. HHP of the almonds was performed for 5 min using pressure/temperature combinations of 50,000 psi/25 °C, 60,000 psi/25 °C or 60,000 psi/50 °C or for 10 min using a pressure/temperature combination of 70,000 psi/55 °C.

2.8.2. Discontinuous process

Fifty gram portions of inoculated, vacuum packaged almonds, were pretempered to 50 °C or 55 °C, and subjected to a discontinuous (oscillatory) hydrostatic pressure process. The discontinuous process consisted of six cycles of pressuring the almonds for 20 sec at a pressure temperature combination of 60,000 psi/50 °C or 70,000 psi/55 °C, followed by holding the almonds for 30 s at 0 psi.

2.8.3. Water pressurization

Twenty five gram portions of inoculated almonds were placed into 500 ml plastic bottles (Nalgene, Rochester, NY), suspended in 500 ml of sterile water pretempered to 25 °C, and subjected to continuous hydrostatic pressure processes. The continuous processes consisted of a treatment at 60,000 psi at a temperature of 25 °C for 5 min. Following water pressurization, the almonds were removed from the bottles, drained of excess water, and allowed to dry at 55 °C using a handheld blow dryer for 5 min. Following the dry step, the almonds were processed as described above.

2.9. Kinetic study of inoculated almonds

To determine the *D* value for reduction of *S.* Enteriditis on almonds during the continuous process, 50 g portions of inoculated almonds were pre-tempered to 50 °C, and processed at 60,000 psi for 0, 3, 5, 7 or 10 min. The *D* value was determined by plotting the log survivors verses time, and determining the inverse of the positive value of the slope. Following all HHP procedures, samples were enumerated as described above.

3. Results and discussion

3.1. Inactivation of S. Enteriditis in pure culture

Bacterial suspensions of *S.* Enteriditis FDA ($\log_{10}$ 7.46) and PT30 ($\log_{10}$ 8.16) in 0.1% peptone were pressurized between 50,000 and 70,000 psi for 5 min at either 25 °C or 50 °C. Immediately after pressurization the vials containing the bacteria were stored at 4 °C and enumerated. Initial pressurization studies concentrated on the ability of HHP to inactivate *S.* Enteriditis FDA. In previous studies, this strain was shown to be highly pressure resistant (Alpas et al., 1999). The results, presented in Table 1 show that a pressure/temperature/time combination of 60,000 psi/25 °C/5 min was sufficient to completely eliminate both *S.* Enteriditis strains.

Table 1
Viability loss of two *Salmonella* Enteriditis strains suspended in 0.1% peptone following pressurization at various pressure and temperature combinations for 5 min

Bacterial strain	HPP (psi)/temperature/time	Log_{10} CFU/ml	Viability loss ($\log_{10}$)
S. Enteriditis (FDA)	0/25 °C/5 min (control)	7.46	–
	50,000/25 °C/5 min	3.00	4.45
	60,000/25 °C/5 min	ND[a]	7.46
	70,000/25 °C/5 min	ND	7.46
	60,000/50 °C/5 min	ND	7.46
S. Enteridtis (PT30)	0/25 °C/5 min (control)	8.16	–
	50,000/25 °C/5 min	6.12	2.04
	60,000/25 °C/5 min	ND	8.16
	70,000/25 °C/5 min	ND	8.16
	60,000/50 °C/5 min	ND	8.16

[a] ND, no CFU were detected in 1 ml of cell suspension from each of the samples tested.

3.2. Inactivation of S. Enteriditis inoculated onto raw almonds using continuous HHP

To initially investigate the ability of HHP to inactivate *S.* Enteriditis on raw almonds, almonds were inoculated with *S.* Enteriditis FDA, and subjected to various pressure/time/temperature combinations (Table 2). The results indicated that pressurizing the almonds at 50,000 psi or 60,000 psi at 25 °C for 5 min, resulted in no loss in viability of the *S.* Enteriditis cells. Pressurizing the almonds at 60,000 psi, and 50 °C for 5 min resulted in a $\log_{10}$ 0.83 reduction in the *S.* Enteriditis concentration. Interestingly, pressurization of the almonds at 70,000 psi and 55 °C for 10 min, only reduced the *S.* Enteriditis concentration by $\log_{10}$ 0.51. The decreased efficacy of HHP at 70,000 psi was also observed during the discontinuous process (Table 3). At 70,000 psi, the surface of the almonds became visibly oily, presumably due to the high pressure forcing oil out of the interior of the almonds. Previous studies have shown that foods with high fat content can exhibit a baroprotective effect on bacterial inactivation by HHP (Gervilla, Ferragut, & Guamis, 1999). In the current study, we speculate that the oil may have had a protective effect on the *S.* Enteriditis cells.

The reduced ability of HHP to inactivate the *S.* Enteriditis cells inoculated onto the raw almonds (as compared to the 0.1% peptone water), was probably due to the low water activity (a_w) of the almonds. Low water activity has been observed to impart baroprotective effects on bacterial cells (Hayert, Perrier-Cornet, & Gervais, 1996). To investigate the effect of low a_w on the *S.* Enteriditis cells during HHP, the a_w of the raw almonds was measured for a 24 h period (immediately before bacterial inoculation and following bacterial inoculation). The results showed that the raw almonds had an initial a_w of 0.556; at 24 h, the water activity had dropped to 0.15. Adjusting the a_w of the almonds to 0.59 led to an increase in the $\log_{10}$ reduction of the *S.* Enteriditis concentration, when the almonds were pressurized at 70,000 psi and 55 °C, for 10 min ($\log_{10}$ 0.75 reduction vs. $\log_{10}$ 0.51 reduction).

Table 2
Viability loss of *Salmonella* Enteriditis FDA inoculated onto raw almonds following continuous pressurization at various pressure and temperature combinations for 5 min

Bacterial strain	HPP (psi)/temperature/time	Log_{10} CFU/ml	Viability loss ($\log_{10}$)
S. Enteriditis (FDA)	0/25 °C/5 min (control)	6.81	–
	50,000/25 °C/5 min	6.76	0.05
	60,000/25 °C/5 min	6.78	0.03
	60,000/50 °C/5 min	5.98	0.83
	70,000/55 °C/10 min	6.30	0.51

Table 3
Viability loss of two *Salmonella* Enteriditis strains inoculated onto raw almonds following a discontinuous pressurization process at 50 °C for 5 min

Bacterial strain	HPP (psi)/temperature/time	Log_{10} CFU/ml	Viability loss ($\log_{10}$)
S. Enteriditis (FDA)	0/50 °C (control)	6.78	–
	60,000/50 °C	5.51	1.27
	0/55 °C (control)	8.00	–
	70,000/55 °C	6.94	1.06
S. Enteridtis (PT30)	0/50 °C (control)	8.18	–
	60,000/50 °C	7.02	1.16

3.3. Inactivation of S. Enteriditis inoculated onto raw almonds using discontinuous HHP

The high barotolerance of the *S.* Enteriditis cells dictated an investigation into different methods of pressure application, in an attempt to determine if bacterial inactivation could be increased. To determine the effect of discontinuous processing on the inactivation of *S.* Enteriditis on raw almonds, the almonds were subjected to a discontinuous process of hydrostatic pressure for 6 cycles at either 60,000 psi or 70,000 psi and 50 °C or 55 °C. Each cycle consisted of holding the pressure vessel at the required pressure for 20 s, followed by holding the vessel at 0 psi for 30 s. The results are indicated in Table 3. For both strains of *S.* Enteriditis, the discontinuous process resulted in greater than a 1 log reduction in viable bacteria. These results are in agreement with the other studies (Hayakawa, Kanno, Yoshiyama, & Fujio, 1994; Palou et al., 1998), that showed that discontinuous processing is more effective than continuous processing at reducing bacterial concentrations. Nevertheless, it is clear that the low a_w of the almonds limited the ability of the discontinuous process to greatly reduce the *S.* Enteriditis concentration, a result supported by other studies (Palou et al., 1998). The manner in which pressure is applied during processing (continuously or discontinuously) has a significant effect on the bacterial inactivation achieved, and several studies have shown that discontinuous pressure processing can vastly increase bacterial inactivation as compared to continuous processing (Palou et al., 1998; Hayakawa et al., 1994).

3.4. Inactivation of S. Enteriditis inoculated onto raw almonds using water pressurization (continuous HHP)

The results suggested that temporarily adjusting the water activity of the almonds followed by pressurization and a drying step would lead to a greater decrease in *S.* Enteriditis concentration on the surface of the raw almonds. In these studies, we used the *S.* Enteriditis strain PT30, since *S.* Enteriditis FDA (used in the studies described above) was found to be not as pressure resistant as *S.* Enteriditis PT30. We developed a water pressurization procedure, in which the almonds were directly suspended in water prior to pressurization, pressurized, and rapidly dried. The rationale behind this strategy was the fact that suspending the almonds in water would increase the a_w at the surface of the almond, allowing the pressure to impart a more destructive effect on the bacteria.

Table 4
Estimated *D* value of *Salmonella* Enteriditis PT30 following continuous pressurization at 60,000 psi and 50 °C

Bacterial strain	Time	Log_{10} CFU/ml	Viability loss (log_{10})
S. Enteriditis (PT30)	0 (control)	8.18	–
	3	7.89	0.29
	5	7.79	0.39
	7	7.30	0.88
	10	7.23	0.95

The *D* value was calculated from the absolute value of the inverse of the slope from linear regression between logarithm of survivors and times.

The results indicated that the water pressurization method resulted in a log_{10} 3.37 reduction in the *S.* Enteriditis concentration when compared to the control (dry, non-inoculated almonds), and a log_{10} 2.73 reduction when compared to a second control consisting of almonds that were suspended in water and dried, but not pressurized. These results show that water pressurization of almonds effectively reduces the *S.* Enteriditis concentration. Studies in this area are ongoing.

3.5. Calculation of the decimal reduction time

The decimal reduction time was calculated for *S.* Enteriditis PT30, since this strain was found to be more resistant than *S.* Enteriditis FDA in the continuous (Table 1) and discontinuous (Table 3) HHP processes. Using the viability loss data in Table 4, the *D* value for inactivation of *S.* Enteriditis inoculated onto raw almonds and pressurized at 60,000 psi and 50 °C was determined to be 9.78 min.

4. Conclusion

HHP processing continues to find increased application within the food industry, although the process is still economically expensive. It is likely that HHP parameters for food pasteurization will have to be developed to ensure reduction in the populations of foodborne pathogens by at least 5 logs.

The results of this study confirmed the results of other researchers (Palou et al., 1998; Hayert et al., 1996), showing that low a_w imparts a protective effect on bacterial cells. This work also showed that HHP is much more effective at reducing the microbial load in foods with higher water activities. Depending on the physical nature of the food, it may be possible to suspend the food to be pressurized directly in the pressurizing medium (water). As shown in this work, such an approach would have the effect of increasing the a_w of the food, leading to increased reduction in the concentration of vegetative bacteria.

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